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Association of TLR4 Mutations and the Risk for Acute GVHD After HLA-Matched–Sibling Hematopoietic Stem Cell Transplantation

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ABSTRACT

Lipopolysaccharide (LPS) has been implicated in the pathogenesis of graft-versus-host disease (GVHD). The toll-like receptor (TLR)-4 has been recently identified as a major receptor for LPS. Mutations of TLR4 have been associated with LPS hyporesponsiveness. We hypothesized that TLR4 mutations reduce the risk of acute GVHD in allogeneic marrow transplant recipients. In a preliminary study to determine the frequency of TLR4 mutations and their possible association with GVHD, we tested 237 patients and their HLA-identical sibling donors for 2 TLR4 polymorphisms. All patients received methotrexate and cyclosporine for GVHD prophylaxis. One or more mutants were detected in 10.8% of patients and 10.6% of donors. Multivariable logistic regression models were used to analyze the association between TLR4 mutations and probability (1-sided) of GVHD. The odds ratio (adjusted for advanced disease, total body irradiation dose, and patient age) for development of grades II to IV GVHD when a mutation was present in the recipient was 0.63 (95% confidence interval [CI], 0.25-1.60; *P* = .16). When a mutation was present in the donor, the adjusted odds ratio was 0.88 (95% CI, 0.36-2.17; *P* = .40). When a mutation was present in both recipient and donor, the odds ratio was 0.72 (95% CI, 0.22-2.32; *P* = .29). Among 24 patients with TLR4 mutations in either donor or recipient, 4 (16.7%) developed gram-negative bacteremia. Among 213 patients without mutations, 14 (6.6%) developed gram-negative bacteremia (*P* = .09). The data indicate that a reduced risk of acute GVHD is associated with TLR4 mutations and that TLR4 mutations may increase the risk for gram-negative bacteremia. However, these associations are not statistically significant in recipients of HLA-matched sibling marrow transplants who are prophylactically treated for infections and GVHD. A much larger study population would be needed to confirm the role of LPS in the pathogenesis of GVHD in humans.

KEY WORDS

HLA • Lipopolysaccharide • Toll-like receptor 4

INTRODUCTION

Acute graft-versus-host disease (GVHD) is a major cause of morbidity and mortality after hematopoietic stem cell transplantation (HSCT). Whereas HLA disparity between donor and recipient is a major GVHD risk factor, bacteria have long been postulated to contribute to the pathogenesis of GVHD [1,2]. A protective environment was found to decrease the incidence of GVHD after marrow transplantation in aplastic anemia patients [3], whereas higher-dose total body irradiation regimens that damage gut mucosa and facilitate translocation of bacteria and bacterial products increased the incidence of GVHD [4]. Gram-negative bacteria and their cell wall component, lipopolysaccharide (LPS), have been implicated in experimental GVHD in murine transplantation models [1]. GVHD in mice was less severe in (C3FeB6)F1 recipients of bone marrow from LPS-resistant (C3H/HeJ) donors compared with recipients of bone marrow from LPS-sensitive (C3HeB/FeJ) donors [5].

The toll-like receptor (TLR)-4 has recently been identified as a signal-transducing component in the LPS receptor complex. C3H/HeJ and C57BL/10ScCr mouse strains with low responses to LPS have mutations in TLR4 [6]. The missense mutation in C3H/HeJ mice is codominant in that heterozygotes have intermediate responses to LPS. A TLR4

knockout mouse recapitulates the LPS-hyporesponsive phenotype [7]. These TLR4-deficient strains are highly susceptible to gram-negative bacteremia [7,8].

Certain TLR4 alleles in humans have now been associated with endotoxin hyporesponsiveness [9]. Two cosegregating polymorphisms in the extracellular domain of the TLR4 receptor were identified in a group of individuals with LPS-hyporesponsive blood monocytes and blunted airway responsiveness to inhaled LPS [10]. Both homozygous and heterozygous genotypes were associated with LPS hyporesponsiveness, with heterozygous genotypes having an intermediate phenotype. Furthermore, these alleles are associated with the development of gram-negative sepsis (E. Lorenz, J.P. Mira, K.L. Frees, D.A. Schwartz, unpublished data, 2001) and more complications from sepsis in severely ill patients (R.R. Schumann, E. Lorenz, E. Latz, N. Limmer, P.M. Schlag, D.A. Schwartz, unpublished data, 2001). The TLR4 Asp299Gly mutation was shown to disrupt TLR-mediated LPS signaling in vitro more severely than the TLR4 Thr399Ile mutation. Transfection of the common wild-type allele of TLR4 rescued the LPS-resistant phenotype in airway epithelial cells and alveolar macrophages from subjects with TLR4 mutations [9].

The objectives of this preliminary study were (1) to determine the frequency of TLR4 mutations in a large cohort of HSCT recipients and their HLA-identical donors and (2) to test the hypothesis that TLR4 mutations in either the recipient or donor reduce the risk of acute GVHD in allogeneic HSCT recipients by minimizing the response to LPS.

MATERIALS AND METHODS

Patient Selection

DNA samples were obtained from a previously described cohort of patients (n = 237) who received an HSCT from an HLA-identical sibling [11]. All patients received methotrexate and cyclosporine for GVHD prophylaxis and had either grade 0 or grades II to IV acute GVHD. Patients with grade I GVHD, patients with renal failure requiring dialysis, and patients without GVHD who died before day 80 after transplantation were excluded.

TLR4 Genotyping

Testing was performed by individuals who did not know which patients had GVHD. Genotyping was performed using allele-specific polymerase chain reaction (PCR) assays for TLR4 Asp299Gly and TLR4 Thr399Ile mutations. In a separate study [12] to determine the reliability of the PCR-based assay, 1 sample each from 10 patients were sequenced and confirmed the results of the PCR-based restriction-fragment length–polymorphism assay. The PE Taq polymerase kit (PE Applied Biosystems, Foster City, CA) was employed for PCR reactions. In a total reaction volume of 25 μL, 2.5 μL of 10× PCR buffer, 20 pmol of each primer (see below), 0.02 μg of genomic DNA, 5 U of Taq polymerase, and 1 μL dNTP (deoxyribonucleoside triphosphate) mix (Clontech, Palo Alto, CA) were combined. Primers for TLR4 Asp299Gly were (F5′GATTAGCAT ACTTAGACTACTACCTCCATG) and (R5′GATCAA CTTCTGAAAAAGCATTCCCAC). Primers for TLR4 Thr399Ile were (F5′GGTTGCTGTTCTCAAAGT GATTTTGGGAGAA) and (R5′CCT GAAGACTGG AGAGTGAGTTAAATGCT). The underlined bases in both forward primers indicate the nucleotide altered to create an NcoI (TLR4 Asp299Gly) and a HinfI (TLR4 Thr399Ile) restriction site, respectively. PCR reactions were run at 95°C for 4 minutes followed by 30 cycles at 95°C for 30 seconds, 55°C for 30 seconds, 72°C for 30 seconds. A 5-μL aliquot of the product was digested with the appropriate restriction enzyme and electrophoresed in a 3% NuSieve gel to identify the TLR4 alleles.

Statistical Analysis

GVHD scores were unblinded after all TLR4 typing was completed. Univariate and multivariate logistic regression models were used to analyze the association between risk factors and the probability of acute GVHD. All *P* values are 1-sided to match the 1-sided hypothesis that GVHD was reduced in the mutant allele groups and all *P* values were derived from the Wald test. No adjustments were made for multiple comparisons.

RESULTS AND DISCUSSION

Among 227 donors who were successfully genotyped, 203 had no detected mutations of TLR4 and 24 (10.6%) had at least 1 TLR4 mutation (Table 1). Among 223 patients who were genotyped, 199 had no TLR4 mutations detected, and 24 (10.8%) had at least 1 TLR4 mutation. Of 24 recipients with TLR4 mutation(s), 14 had donors with TLR4 mutation(s). The allelic frequency for the 299 and 399 polymorphisms were 4.7% and 5.3%, respectively, in both the donor and recipient populations which is in agreement with the cosegregate nature of the *TLR*4 mutation. These populations were in Hardy-Weinberg equilibrium.

Grades II to IV acute GVHD occurred in 94 (47.2%) of 199 recipients with no TLR4 mutations and in 8 (33.3%) of 24 recipients with any TLR4 mutations (*P* = .10) (Table 1). Acute GVHD occurred in 96 (47.3%) of 203 recipients whose donors had no TLR4 mutations and in 10 (41.7%) of 24 recipients whose donors had any TLR4 mutations (*P* = .30). From 14 donor-recipient pairs in which both the donor and recipient had TLR4 mutations, 5 (35.7%) patients had acute GVHD compared to 89 (49.2%) of the patients from 181 pairs in which neither the donor nor the recipient had TLR4 mutations (*P* = .17). Because of the small numbers, further analysis according to specific mutations was not performed. Moreover, LPS hyporesponsiveness of varying degrees is expected in both homozygous and heterozygous individuals [9].

Analysis by logistic regression models gave qualitatively similar results (Table 2). In this analysis, we also adjusted for other previously determined risk factors for acute GVHD including patient age at transplantation, dose of total body irradiation, and advanced malignancy [11]. The estimated risks for acute GVHD expressed as an odds ratio was less than 1.0 when TLR4 mutations were present, but the associations were statistically modest.

TLR4 mutations are associated with increased susceptibility to gram-negative bacteremia in mice and patients [8] (R.R. Schumann, E. Lorenz, E. Latz, N. Limmer, P.M. Schlag, D.A. Schwartz, unpublished data, 2001). We also

Table 1. *Association of TLR4 Mutations and Acute GVHD**

Donor TLR4 Genotype†		Acute GVHD Score			
299	399	Grade 0	Grade II	Grade III	Grade IV
00	00	107	60	33	3
00	01	1			
01	00	1			
01	01	12	7	2	
11	11				1
Patient TLR4 Genotype†		**Acute GVHD Score**			
299	399	Grade 0	Grade II	Grade III	Grade IV
00	00	105	59	32	3
00	01	1			
01	00	2			
01	01	12	5	2	
11	01				1
11	11	1			

*Acute GVHD score data are n in each category. TLR indicates toll-like receptor; GVHD, graft-versus-host disease.

†TLR4 genotypes corresponding to Asp299Gly and Thr399Ile as described in text; 0 indicates no mutation; 1, mutation.

detected a trend for a similar association in this cohort of patients, even though they were routinely treated with antibiotics during neutropenia. In this cohort, 18 patients had gram-negative bacteremia. Among 24 patients with TLR4 mutations in either donor or recipient, 4 (16.7%) developed gram-negative bacteremia. Among 213 patients without TLR4 mutations, 14 (6.6%) developed bacteremia ($P = .09$). The odds ratio was 2.44 (95% CI, 0.87-6.82).

LPS has been postulated to contribute to the pathogenesis of GVHD. One paradigm involves translocation of bacteria or bacterial products across gut mucosa. LPS may then prime macrophages for augmented production of effector molecules (eg, tumor necrosis factor α, nitric oxide) in response to donor T-cell proinflammatory cytokines. In murine transplantation models, endotoxemia and macrophage activation are associated with GVHD [1]. Transplantation of marrow from donor mice with mutated TLR4 (C3H/HeJ) reduced the severity of GVHD [5]. The biological effect of TLR4 mutations observed in murine marrow transplantation models of GVHD [5] might be obscured in humans by posttransplantation immunosuppression with methotrexate and cyclosporine and by antibiotic prophylaxis during neutropenia. LPS has prominent effects on hematopoietic cells, especially those of monocyte-macrophage lineage. These cells have been considered as important effectors of LPS-augmented responses in experimental models of GVHD [1]. Accordingly, we anticipated that protection from GVHD would come primarily from TLR4 mutations in the donor. Our results suggest that protection could come from TLR4 mutations in either the donor or the recipient. Endothelial cells and fibroblasts in the recipient are responsive to LPS in the presence of soluble CD14

Table 2. *Logistic Regression Models of TLR4 Mutation as a Risk Factor for Grades II to IV GVHD**

	Unadjusted Model			Adjusted Model†		
TLR4 Genotype‡	Odds Ratio	95% CI	*P*	Odds Ratio	95% CI	*P*
Patient						
No mutation	1	—	—	1	—	—
Mutation	0.56	0.23-1.36	.10	0.63	0.25-1.60	.16
Donor						
No mutation	1	—	—	1	—	—
Mutation	0.80	0.34-1.88	.30	0.88	0.36-2.17	.40
Patient and donor§						
No mutation	1	—	—	1		
Mutations	0.57	0.19-1.78	.17	0.72	0.22-2.32	.29

*TLR indicates toll-like receptor; GVHD, graft-versus-host disease; CI, confidence interval.

†Adjusted for patient age, dose of total body irradiation, and advanced disease.

‡TLR4 mutations include any mutation as described in the text.

§Analysis included only cases in which donor and recipient had concordant presence or absence of mutations.

[13] and may contribute to GVHD. Adding to the complexity of genetic susceptibility to GVHD is the recognition that polymorphic alleles of other TLR family members and cytokine genes may influence the recognition of and subsequent inflammatory response to bacterial products [14,15].

Studies to assess the role of TLR4 and other members of the toll-like receptor family on GVHD and other important end points such as chronic GVHD, infections, and survival will be limited by the relatively small number of patients and donors with mutations. This small study population limits the power to detect differences that might be considered to be clinically significant, because studies powered to detect such differences will necessarily be quite large. For example, if one were to design a cohort study to assess the association of mutation rate with GVHD based on the observed data from this study (mutation rate of 10%, GVHD rate of 50%), 1500 patients would be needed to achieve 90% power to detect an odds ratio of 0.6 at the 1-sided significance of 0.05. Applying more stringent statistical tests (eg, 2-sided significance test) would further increase the required cohort size and decrease the feasibility of this approach. Other study designs such as case-control analyses might be a more productive approach to examining the susceptibility to GVHD associated with TLR4 alleles and other genetic polymorphisms in proinflammatory pathways.

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